AWARD NUMBER: W81XWH-16-1-0076

TITLE: CB2 Receptor Therapy Using the FDA-Approved Drug Raloxifene to Mitigate Visual Deficits after Mild TBI and/or Ocular Trauma

PRINCIPAL INVESTIGATOR: Anton Reiner

CONTRACTING ORGANIZATION: University of Tennessee Health Science Center
Memphis, TN 38163

REPORT DATE: April 2018

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
April 2018	Annual	15 Mar 2017 - 14 Mar 2018

4. TITLE AND SUBTITLE
CB2 Receptor Therapy Using the FDA-Approved Drug Raloxifene to Mitigate Visual Deficits after Mild TBI and/or Ocular Trauma

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-1-0076

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Anton Reiner

E-Mail: areiner@uthsc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Tennessee Health Science Center
Memphis, TN 38163

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT .
Visual deficits after traumatic brain injury (TBI) or after non-rupturing ocular trauma are highly common in the military, but interventions that limit the post-trauma visual impairments have not been identified. We have found that treatment with a CB2 cannabinoid receptor inverse agonist for 2 weeks after closed-head blast TBI greatly attenuates the visual deficits and retinal pathology produced by mild traumatic brain injury in mice, apparently by modulating the otherwise deleterious role of microglia in the injury process after trauma. The drug we have used (SMM189), however, has not yet been approved for human use. Raloxifene is an FDA approved estrogen receptor drug that is used to treat osteoporosis, but was recently found to show noteworthy CB2 inverse agonism. In the current studies, we are testing the benefit of raloxifene for reducing visual deficits and retinal and optic nerve damage from mild TBI and closed-globe ocular injury in mice. In the second year of the project, we have been determining if raloxifene reduces visual deficits and pathology, when delivered daily after TBI produced using our focal cranial blast injury or an impact injury model. Visual system injury and its abatement with raloxifene is being assessed by functional testing (visual acuity, contrast sensitivity, the scotopic electroretinogram, pupil light response, and light aversion) and morphological analysis of retina, optic nerve, optic tract, central visual structures and oculomotor nerves. We have found that raloxifene for both TBI models does reduce visual deficits, and for blast we have completed studies showing it abates light aversion, and mitigates the underlying visual system pathology and inflammatory response, seemingly by modulating microglia to a more beneficial state. If our proposed animal studies continue to show benefit of raloxifene in preventing visual deficits and injury after brain and/or ocular trauma, it could be next tested in phase 2 human clinical trials to determine its efficacy in treating visual injury after brain and/or eye trauma, speeding its eventual approval for the use in military trauma victims. In that context, it could be adopted as a routine treatment administered by medical personnel shortly after trauma, and thereby prevent or reduce the harmful consequences of the trauma for vision.

15. SUBJECT TERMS
traumatic brain injury, ocular blast injury, raloxifene, CB2 receptors, CB2 inverse agonist, visual system, retina, optic nerve, microglia, neuroinflammation

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	UU	28	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Introduction

Visual deficits after traumatic brain injury (TBI) or after non-rupturing ocular trauma (by itself or as a TBI comorbidity) are highly common in the military, often leading to inability to return to service and/or life-long impairments. Interventions that limit the post-trauma visual impairments, however, have not been identified. We have found that treatment with a cannabinoid type-2 receptor (CB2) inverse agonist for 2 weeks after closed-head blast TBI greatly attenuates the visual deficits and retinal pathology produced by mild traumatic brain injury in mice, apparently by modulating the otherwise deleterious role of microglia in the injury process after trauma. The drug we have used (SMM189), however, has not yet been approved for human use. Raloxifene is an FDA approved estrogen receptor drug that is used to treat osteoporosis, but was recently found to also show noteworthy CB2 inverse agonism. In the current studies, we are testing the benefit of raloxifene for reducing visual deficits and retinal and optic nerve damage from mild TBI and closed-globe ocular injury in mice. In the case of TBI, our plan is to use two different standardized models - our focal blast model of mild TBI in the first year of the project, and an impact model of mild TBI that also yields optic nerve injury in the second year. Our goal is to determine if raloxifene reduces visual deficits and pathology, when delivered daily after TBI produced in both cases. In the case of ocular blast injury (year 3 of project), our goal is likewise to determine if raloxifene reduces visual deficits and pathology, when delivered daily after the trauma. Visual system injury and its abatement with raloxifene is being assessed by functional testing (acuity, contrast sensitivity, the scotopic electroretinogram, the pupil light response, and light aversion) and morphological analysis of retina, optic nerve, optic tract, central visual structures and oculomotor nerves. In addition, morphological and biochemical analysis is being used to assess the role of microglial biasing from the harmful M1 phenotype toward the protective M2 phenotype in raloxifene benefit. Effective raloxifene dose and duration of treatment is being determined as well. If our proposed animal studies show benefit of raloxifene in preventing visual deficits and injury after brain and/or ocular trauma, it will next be tested in phase 2 human clinical trials to determine its efficacy in treating visual injury after brain and/or eye trauma, speeding its eventual approval for the use in military trauma victims. In that context, it could be adopted as a routine treatment administered by medical personnel shortly after trauma, and thereby prevent or reduce the harmful consequences of the trauma for vision, as well as perhaps for cognition and mood.

Keywords

Traumatic Brain Injury
Ocular Blast Injury
Raloxifene
CB2 receptors
CB2 receptor inverse agonist
Visual system
Retina
Optic nerve
Visual acuity
Electroretinogram (ERG)
Pupil light response
Light aversion
Microglia
Neuroinflammation

Accomplishments

Our efforts during the second year of the project focused on completing the studies of Task 1 (Aim 1), and pursuing studies of Task 2 (Aim 2). The studies of Task 1 are now nearly complete, and those of Task 2 should be completed this summer. The completed studies from both Task 1 and Task 2 show the benefit of raloxifene in reducing visual deficits after TBI. We separately discuss progress below on these two Tasks, beginning with Task 1 in each case.

Major Goals for the First Year of the Project as listed in SOW:

Overall Major Task 1: Determine if raloxifene alleviates the visual deficits and causative neural abnormalities in a mouse blast model of mild TBI (Aim 1). This Task was divided into three subtasks as described below.

Subtask 1-1: Determine if raloxifene reduces the visual deficits and pathology in our focal blast model of mild TBI, when delivered daily after the TBI event. This was to be carried out in the first 6 months of the project. We have in fact shown that raloxifene reduces visual deficits and pathology in our focal blast model of mild TBI. Some of this functional analysis was completed during year 2 of the project, as detailed below. The pathology studies were also largely completed during year 2, as also detailed below. We estimate this subtask is 95% complete.

Subtask 1-2: Determine if raloxifene benefit in our focal blast model of mild TBI stems from modulation of microglia to the beneficial M2 phenotype. This task was to be completed during the second 6 months of the project. It was initiated during the second six months, and is currently approximately half complete.

Subtask 1-3: Determine treatment window for the raloxifene benefit in our focal blast model of mild TBI. This was to be completed during the last 2 months of year 1, and was completed 100% during year 2 of the project, as detailed below.

Major Goals for the Second Year of the Project as listed in SOW:

Overall Major Task 2: Determine if raloxifene alleviates the visual deficits and causative neural abnormalities in a mouse impact model of mild TBI (Aim 2). This Task was divided into three subtasks as described below.

Subtask 2-1: Determine if raloxifene reduces the visual deficits and pathology in a focal impact model of mild TBI, when delivered daily after the TBI event. This was to be carried out in the first 6 months of year 2 of the project. We have in fact shown that raloxifene reduces visual deficits in our focal blast model of mild TBI, as detailed below. Some of the functional studies have been completed and others are underway and will be completed shortly. The pathology studies will be completed this summer. We estimate this subtask is 40% complete.

Subtask 2-2: Determine if raloxifene benefit in a focal blast impact of mild TBI stems from modulation of microglia to the beneficial M2 phenotype. This task was to be completed during the second 6 months of the second year of the project. These studies will be performed once the similar studies of Subtask 1-2 are completed.

Subtask 2-3: Determine treatment window for the raloxifene benefit in a focal impact model of mild TBI. This will be completed during the first six months of year 3.

What was accomplished under these goals?

We discuss accomplishments under Tasks 1 and 2 below, beginning with Task 1 in each case.

Task 1

1) Major Activities. We have conducted behavioral, electrophysiological, morphological and biochemical studies in mice to evaluate the benefit of raloxifene in alleviating visual deficits and visual system injury after mild TBI produced using our blast system (Subtask 1-1). We have evaluated raloxifene benefit for the following visual deficits or abnormalities after blast mild TBI:

1) a reduction in visual acuity; 2) a reduction in contrast sensitivity; 3) a reduction in the A-wave of the scotopic ERG; 4) a reduction in the B-wave of the scotopic ERG; 5) an increase in light aversion; and 6) abnormality in the pupil light reflex. These studies were initiated in the first year and have been completed during the second year. Morphological studies have assessed and will continue to assess the rescue by raloxifene of structural damage to the visual system by blast mild TBI (Subtask 1-1). Neurochemical and biochemical studies have assessed and will continue to assess the modulatory influence of raloxifene on microglia – in particular the ability of raloxifene to bias microglia from the harmful M1 phenotype to the helpful M2 phenotype (Subtask 1-2). Functional and morphological studies have assessed the effective treatment window for raloxifene (Subtask 1-3).

Task 2

1) Major Activities. We are conducting behavioral, electrophysiological, and morphological studies in mice to evaluate the benefit of raloxifene in alleviating visual deficits and visual system injury after mild TBI produced using an impact system (Subtask 2-1). We are conducting ongoing studies that are partially complete to evaluate raloxifene benefit for the following visual endpoints after impact mild TBI: 1) visual acuity (completed during the second year); 2) contrast sensitivity (completed during the second year); 3) the A-wave of the scotopic ERG; 4) the B-wave of the scotopic ERG; 5) light aversion (partly completed during the second year); and 6) the pupil light reflex (partly completed during the second year). Morphological studies will assess the rescue by raloxifene of structural damage to the visual system by blast mild TBI (Subtask 2-1). Our studies show that impact TBI does injure optic nerve. Neurochemical and biochemical studies will assess the modulatory influence of raloxifene on microglia – in particular the ability of raloxifene to bias microglia from the harmful M1 phenotype to the helpful M2 phenotype (Subtask 2-2). Functional studies will assess the effective treatment window for raloxifene (Subtask 2-3).

Task 1

2) Specific Objectives. Our objective was to show by behavioral, physiological, morphological and biochemical studies in mice that raloxifene alleviates visual deficits and visual system injury after mild TBI created using our focal blast model (Subtask 1-1). In the case of behavioral and physiological assessments, we have sought to show that raloxifene alleviates: 1) a reduction in visual acuity following blast mild TBI; 2) a reduction in contrast sensitivity following blast mild TBI; 3) a reduction in the A-wave of the scotopic ERG following blast mild TBI; 4) a reduction in the B-wave of the scotopic ERG following blast mild TBI; 5) an increase in light aversion following blast mild TBI; and 6) an abnormality in the pupil light reflex following blast mild TBI. In the case of our morphological studies, we have sought to show that raloxifene rescues structural damage to the visual system caused by blast mild TBI (Subtask 1-1). In the case of the modulatory influence of raloxifene on microglia, we have sought to provide neurochemical and biochemical evidence that raloxifene converts microglia from the harmful M1 phenotype to the helpful M2 phenotype (Subtask 1-2). Functional and morphological studies have sought to determine the effective treatment window for raloxifene (Subtask 1-3).

Task 2

2) Specific Objectives. Our objective was to show by behavioral, physiological, morphological and biochemical studies in mice that raloxifene alleviates visual deficits and visual system injury after mild TBI caused by an impact device (Subtask 2-1). In the case of behavioral and physiological assessments, we have sought to determine if raloxifene shows a benefit for reducing TBI-induced abnormalities in the following endpoints: 1) visual acuity; 2) contrast sensitivity; 3) the A-wave of the scotopic ERG; 4) the B-wave of the scotopic ERG; 5) light aversion; and 6) the pupil light reflex. In the case of our morphological studies, our goal is to determine if raloxifene rescues structural damage to the visual system caused by impact mild TBI (Subtask 2-1). In the case of the modulatory influence of raloxifene on microglia, our goal is

to provide neurochemical and biochemical evidence that raloxifene converts microglia from the harmful M1 phenotype to the helpful M2 phenotype (Subtask 2-2). Functional studies will determine the effective treatment window for raloxifene (Subtask 2-3).

3) Significant Results or Key Outcomes. In the description below, we review our findings that raloxifene did improve the functional outcome from blast TBI, and that a dose of 10 mg/kg produces a better outcome than does a dose of 5 mg/kg for some endpoints, and the raloxifene benefit is present with treatment over the first 3-6 days postblast. We also review evidence of morphological benefit and the microglial activity of raloxifene in blast TBI. Findings from the second year are described in detail, while findings from year 1 are included for completeness, but only summarized. For each endpoint below, we also describe progress on the same endpoint in impact TBI studies during year 2.

Subtask 1-1: Functional and Morphological Studies of Blast TBI

3A. Raloxifene Benefit – Visual Acuity and Contrast Sensitivity after Blast TBI. In an initial cohort of 10 mice per group during year 1, we found that raloxifene at 5 mg/kg and/or 10 mg/kg reduced post-blast TBI deficits in visual contrast sensitivity and visual acuity, reduced TBI-induced light aversion, and/or mitigated a pupil hyper-responsivity to light. Some of the effects were, however, borderline in their significance. During period 5 (first period of year 2), we therefore began an additional cohort of 5-7 0-psi mice treated with vehicle, 50-psi mice treated with vehicle, and 50-psi mice treated with 5 mg/kg raloxifene. It is important to note that in our blast model, a focal blast is delivered to the left side of the cranium. Our results indicate that this yields somewhat different outcomes for visual function mediated by the two eyes. In any case, the mice in the cohort begun during period 5 were pre-blast tested for visual contrast sensitivity and visual acuity, and were post-blast tested for contrast sensitivity and acuity one month after blast. As in our prior studies, drug or vehicle treatment began within 2 hours of blast and then was continued daily for an additional 2 weeks. These studies represented a completion of the functional studies of contrast sensitivity and visual acuity in Subtask 1 for Aim 1. As shown in graph 1A below, for the total set of 16 50-psi mice that received vehicle compared to the 15 0-psi mice that received vehicle, a significant deficit was seen for both eyes in contrast sensitivity (asterisks in graph in Figure 1A), with the 50-psi vehicle-treated mice significantly worse (i.e. higher % contrast needed). By contrast, for the total set of 17 50-psi mice that received 5 mg/kg raloxifene, no significant difference from sham was seen for either eye in contrast sensitivity compared to the 15 0-psi mice that received vehicle. Moreover, the contrast sensitivity scores for the 50-psi mice that received 5 mg/kg raloxifene trended toward being significantly lower (better) than in the 50-psi vehicle-treated mice (pound sign) for both the left eye ($p=0.0632$) and the right eye ($p=0.0504$). Thus, 50-psi blast produced a significant bilateral deficit in contrast sensitivity at one month after blast, and two weeks of raloxifene beginning 2 hours after blast alleviated this deficit. In the case of the 10 mice receiving 50-psi blast and two weeks of raloxifene beginning 2 hours after blast, a similar benefit as for 5 mg/kg was seen. No significant difference was seen for either eye in contrast sensitivity for the 10 mg/kg mice compared to the 15 0-psi mice that received vehicle, and the contrast sensitivity scores for 50-psi mice that received 10 mg/kg raloxifene were significantly lower (better) than in 50-psi vehicle-treated mice for the right eye. In the case of visual acuity (graph in Figure 1B), a trend (pound sign) for the right eye toward a slight reduction ($p=0.0519$) was seen in the 16 50-psi mice that received vehicle compared to the 15 0-psi mice that received vehicle. Moroever, paired t-tests revealed that acuity declined significantly ($p=0.0309$) in the 50-psi but not the 0-psi mice between the pre-blast test to the post-blast test. By comparison, no acuity deficit was seen for the right eye in either the 50-psi mice that received 5 mg/kg raloxifene or 10 mg/kg raloxifene, either by comparing to the sham blast mice, or by comparing pre-blast and post-blast performance for the 50-psi mice that received 5 mg/kg raloxifene or 10 mg/kg raloxifene. No

acuity deficit or trend toward one was seen in the left eye of 50-psi mice that received vehicle, or in either of the raloxifene-treated blast groups. These overall results for acuity show that, as for contrast sensitivity, raloxifene at either dose alleviated the functional deficit caused by 50-psi blast alone. These results are illustrated in the below graphs.

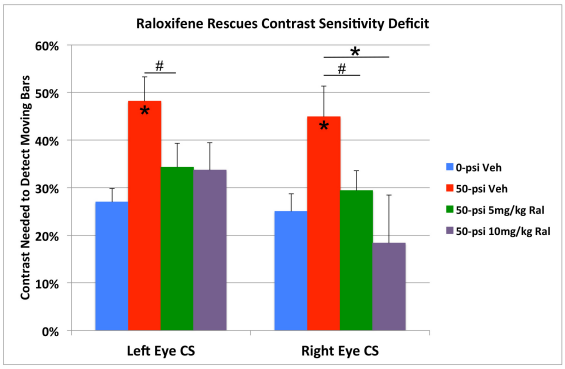


Figure 1A. Contrast sensitivity as measured using Optomotry in sham blast mice, blast mice, and blast mice receiving either raloxifene at 5 mg/kg or 10 mg/kg. The contrast needed to detect the moving stripes was significantly more after TBI for both eyes (asterisk), but not in TBI mice receiving raloxifene. Contrast sensitivity in the 5 mg/kg raloxifene mice was significantly better than in the blast mice with vehicle for both eyes (bar with asterisk), and for the right eye in the case of 10 mg/kg raloxifene.

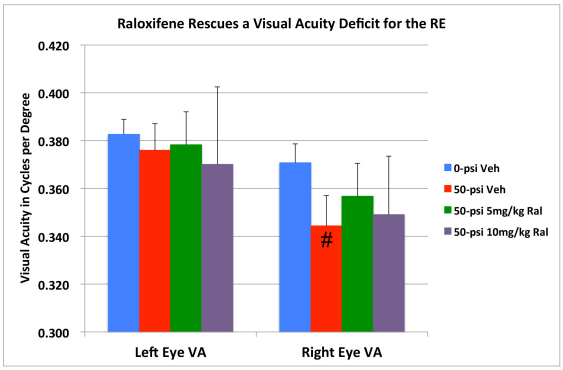


Figure 1B. Visual acuity as measured using Optomotry in sham blast mice, blast mice, and blast mice receiving either raloxifene at 5 mg/kg or 10 mg/kg. There was no deficit in acuity for the left eye. For the right eye, acuity was worse in blast-vehicle mice than in sham mice, as shown by a significant worsening from pre to post blast for the blast-vehicle mice but not the sham mice (pound sign). Acuity in the 5 mg/kg and 10 mg/kg raloxifene blast mice did not differ from sham nor did they change significantly from pre to post blast for the right eye.

Subtask 2-1: Functional and Morphological Studies of Impact TBI

3A. Raloxifene Benefit – Visual Acuity and Contrast Sensitivity after Impact TBI. We assessed visual acuity and contrast sensitivity deficits after impact TBI and their amelioration by raloxifene in 16 sham impact mice treated with vehicle, 21 impact TBI mice treated with vehicle, and 18 impact mice treated with 5 mg/kg raloxifene. Treatment was begun two hours after impact and then daily for two weeks thereafter. TBI was produced in anesthetized mice using an Impact One Stereotaxic Impactor (Leica Biosystems, Buffalo Grove, IL) at a strike velocity of 5 m per second, strike depth of 1.0 mm, and dwell time of 200 milliseconds, to the shaved mouse head at 1.5 mm caudal to Bregma and centered on the midline. Note that as the injury is at the midline, the eyes, optic nerves, and two sides of the brain are equally affected. Thus, data are presented separately for each eye per group and for the two eyes pooled within group.

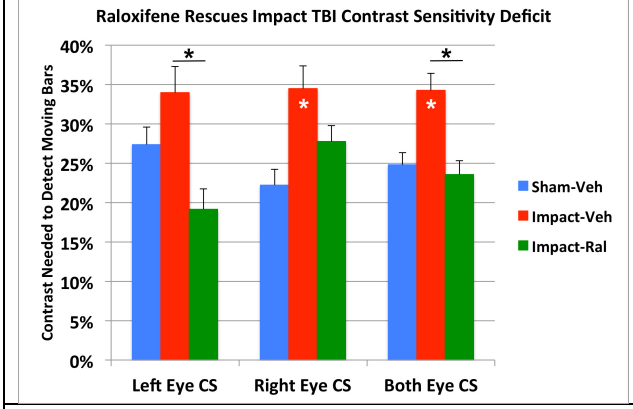


Figure 2A. Contrast needed to detect the moving stripes was significantly more after TBI for the two eyes pooled (asterisk), but not in TBI mice receiving raloxifene. Contrast sensitivity in the raloxifene mice was significantly better than in the blast mice with vehicle.

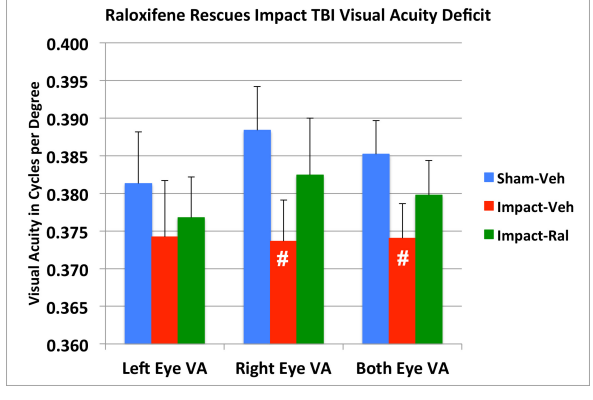


Figure 2B. Visual acuity trended toward being less after TBI for the two eyes pooled (pound sign) than in sham mice, and also less than in TBI mice receiving raloxifene. As in the case of contrast sensitivity, impact TBI yielded a lesser deficit than did blast TBI.

We found that impact TBI yielded lesser deficits than did blast TBI. We do not know if this reflects an inherent difference between blast and impact as concussive forces, or (more likely) to differences between the site of impact (lateral left cranial for blast and dorsal midline cranial for impact) or between the magnitude of the concussive forces. Focusing on the pooled eye data, impact TBI yielded a significant deficit in contrast sensitivity (increased contrast needed to detect the moving stripes) in the impact-vehicle mice ($p=0.0014$), but not in the impact-raloxifene mice ($p=0.5733$). Contrast sensitivity was significantly better in the impact-raloxifene mice than in the impact-vehicle mice ($p=0.0003$). Thus, as with blast TBI, raloxifene rescued a post-TBI contrast sensitivity deficit when treatment was begun two hours after impact and then daily for two weeks thereafter. In the case of visual acuity, a reduction was observed in the impact-vehicle mice compared to the sham-vehicle mice that trended toward but did not achieve significance when compared between groups. Visual acuity was largely the same as in sham in the case of eyes from impact TBI mice treated with raloxifene.

Subtask 1-1: Functional and Morphological Studies of Blast TBI
3B. Raloxifene Benefit – Scotopic Light-adapted ERG A-wave and B-wave after Blast TBI. We measured the A-wave and B-wave amplitudes before and about 8 weeks after blast in the first cohort of blast mice, and compared the relative direction of pre to post changes for the four groups of mice. The studies were completed during Year 1 of the project. No significant preblast differences were seen in any of the four groups for the A-wave or B-wave amplitudes for either eye. The results show that the mean postblast A-wave amplitudes were similarly reduced in both eyes in the 50-psi vehicle (50V) mice compared to the sham-vehicle (0V) mice at the brightest light intensities, and that both doses of raloxifene yielded prominent rescue of this A-wave deficit. The ERG results also show that the average postblast B-wave amplitudes were reduced in the left eyes in the 50V mice compared to the 0V mice across all light intensities, and that both doses of raloxifene yielded prominent rescue of this B-wave deficit. The results were described in detail in the Progress Report for Year 1. Our data thus show that raloxifene at both doses rescued ERG deficits stemming from blast TBI. These results show that, although the eyes were not in direct line with the blast, the pressure wave traversing the skull did affect both retinas, including photoreceptors as revealed by the A-wave data and bipolar cells as revealed by the B-wave data.
Subtask 2-1: Functional and Morphological Studies of Impact TBI
3B. Raloxifene Benefit – Scotopic Light-adapted ERG A-wave and B-wave after Impact TBI. We are assessing the effect of impact TBI and raloxifene on the scoptopic ERG In a current cohort of 11 sham impact mice treated with vehicle, 12 impact TBI mice treated with vehicle, and 12 impact mice treated with 5 mg/kg raloxifene. Pre-TBI testing has been performed during the second project year, and no differences were observed between groups. Mice will be assessed for ERG during the next month to determine if impact TBi adversely affected either the A-wave data or B-wave, and if raloxifene rescued any deficits.

Subtask 1-1: Functional and Morphological Studies of Blast TBI
3C. Raloxifene Benefit – Light Aversion after Blast TBI. Our light-dark test system consists of a clear-walled test arena containing two equally sized compartments – an open area surrounded by the clear Plexiglas walls of the overall test arena and an enclosed chamber with black Plexiglas walls. An opening leads from one to the other. The enclosed chamber, however, contains a light bulb that can illuminate the enclosed chamber at 500 lux, 1000 lux or 1500 lux. Otherwise the enclosed chamber is at 0 lux. The entire test arena is covered by a black drape during the testing, to prevent the mouse from being distracted by room cues or room lighting. The illumination of the outer open area is slightly greater than 0 lux when draped and depends on the illumination of the enclosed chamber (ranging from 2-10 lux accordingly as illumination of the enclosed chamber is increased). Mice tend to spend more time in the

enclosed chamber under normal circumstances, and yet more when anxious, as the enclosed chamber is essentially a place the mouse can hide. For the test, the mouse is placed in the open side and the arena covered with the drape. Infrared laser beams detect mouse movement and location, and a program automatically measures how much time the mouse spends in the open versus enclosed part of the arena. The test begins with 5 minutes of no light in the enclosed chamber (0 lux), followed by 5 minutes each of 500, 1000 and 1500 lux. The idea is that since mice are averse to the bright light, they will spend less time in the enclosed chamber as illumination in it increases. If they have become light averse due to TBI, this trend will be enhanced. We performed this test at different times after blast in sham mice receiving vehicle, TBI mice receiving vehicle, and TBI mice receiving raloxifene. The data used is % time in the enclosed chamber in all cases. In this test, an increased tendency to remain in the enclosed chamber despite its light intensity in particular reflects an anxiety increase.

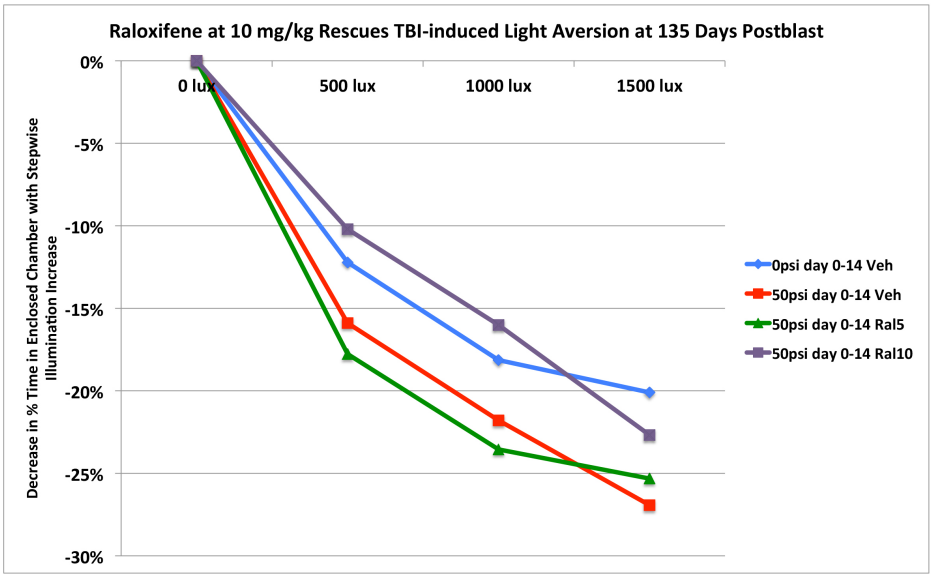


Figure 3. Light aversion is seen in blast mice that received vehicle in a light – dark test, which evaluates reduction in occupancy of an enclosed chamber as the illumination in it increases. Blast-vehicle mice reduced occupancy as light increased to a great extent than did sham-vehicle mice. Raloxifene at 10 mg/kg but not at 5 mg/kg rescued this light aversion.

We evaluated Light - Dark results for blast TBI mice at about 100 and 135 days postblast, and completed the studies during the second year of the project. At 100 days after blast, the 50-psi vehicle-treated mice showed a significant increase in time spent in the enclosed chamber compared to the sham mice, irrespective of its illumination. Any light aversion in response to the illumination of the enclosed chamber was apparently not enough to override the anxiety caused by 50-psi TBI that drives the mice to remain in the enclosed chamber. Notably, raloxifene at 5 mg/kg rescued this anxiety. We then completed a second round of light-dark testing one month after the first for the cohort begun during period 5. Combining the cohorts begun during periods 2 and 5 at 135 days postblast, the results show that the 50-psi vehicle mice no longer showed anxiety compared to the sham mice. The results for the combined cohorts (15-17 total per group) at 135 days after blast showed light aversion in the 50psi vehicle-treated mice. This is seen in the graph above (Figure 3), in which we subtracted time in the enclosed chamber for each light level for each group from enclosed chamber occupancy when the lux in the enclosed chamber was 0. This plot then shows the reduction in enclosed chamber occupancy with increasing illumination, and it can be seen that the 50-psi vehicle-treated mice spent less time in the enclosed chamber than the sham mice as light increases. This light aversion was, however, not rescued by 2-week treatment with 5mg/kg raloxifene. By contrast, it was rescued with 10 mg/kg raloxifene, as the reduction in enclosed chamber occupancy as lighting in it increased was the same for the 10 mg/kg raloxifene blast mice as for the sham mice. Thus, 50-psi blast does cause light aversion by 135 days after blast, and 10

mg/kg raloxifene is needed to prevent this. For these studies, treatment began 2 hours after blast, and then continued daily until day 14 post blast.

Subtask 2-1: Functional and Morphological Studies of Impact TBI

3C. Raloxifene Benefit –Light Sensitivity in Impact TBI Mice. We evaluated Light-Dark Box behavior for impact TBI mice at 70 and 115 days post-TBI during the second project period. Three groups have been analyzed: sham – vehicle mice (n=6), impact TBI – vehicle mice (n=9), and impact - raloxifene mice 5 mg/kg (n=5). After sham or impact TBI, mice received their assigned injections 2 hours after TBI, and then daily for the next 14 days. At 70 days post-TBI (see Figure 4A below), the TBI-vehicle mice spent slightly less time in the enclosed box at 0 lux than the sham mice. Since increased anxiety is associated with increased enclosed box occupancy at 0 lux (as seen in the case of blast TBI at 100 days post blast), these results for impact TBI suggest that the impact – vehicle mice did not show increased anxiety, and perhaps even showed reduced anxiety at 70 days after TBI. As illumination in the enclosed chamber increased, however, they showed less of a light aversion than sham mice, as shown in both figures below. Thus, the impact-vehicle mice at 70 days post-impact appeared to show diminished light aversion, perhaps due to diminished light sensitivity caused by the TBI. Note, however, that the TBI-raloxifene mice were much the same as the sham mice in their response to increasing illumination. Thus, raloxifene at the 5 mg/kg dose rescued the presumptive diminished light sensitivity caused by impact TBI at 70 days after the impact TBI. A second cohort of impact mice has been prepared and will be due for their 70-day post TBI light – dark test May 2018.

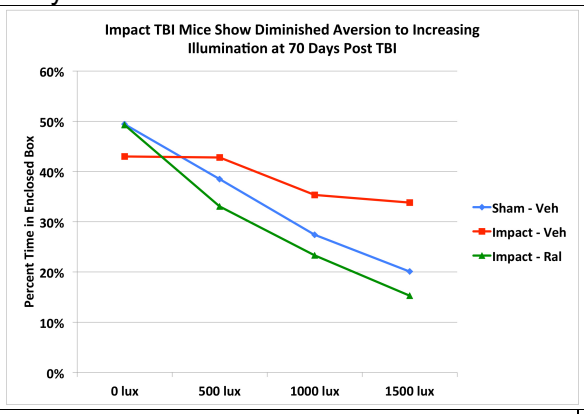


Figure 4A. Impact TBI mice receiving vehicle showed reduced light sensitivity at 70 days post TBI as assessed in a light – dark test, which evaluates reduce occupancy of an enclosed chamber as the illumination in it increases. The raloxifene-treated TBI mice were, however, similar to sham mice in their light aversion. The above graph shows enclosed chamber occupancy as a function of its illumination. Sham and raloxifene-treated TBI mice substantially reduce occupancy as illumination increases.

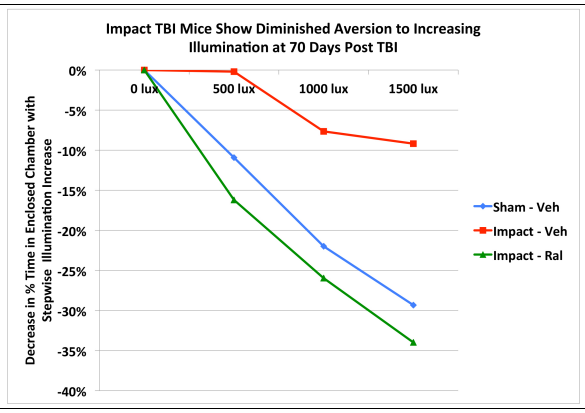


Figure 4B. The above graph shows the same results as in Figure 4A, except in this case it plots reduction in enclosed chamber occupancy as its illumination increases. This plot clearly shows that sham and raloxifene-treated TBI mice substantially reduce occupancy as illumination increases. By contrast, the impact TBI – vehicle mice show much less light aversion and minimally reduce the enclosed chamber occupancy as the light level increases.

At 115 days post TBI, the lighted chamber occupancy was very similar for all groups at 500, 1000, and 1500 lux. Somewhat oddly, the sham mice at this time post impact showed increased anxiety when the chamber was not illuminated, as they spent more time than impact mice in the enclosed chamber at 0 lux. The second cohort of sham and impact TBI mice will help determine if the sham mice consistently show increased anxiety at 0-lux at this time post blast. In any case, the results at this time point show that the diminished light sensitivity seen at 70 days post TBI in the impact – vehicle mice was largely resolved, as the impact - raloxifene and impact - vehicle mice showed similar occupancy of the enclosed camber at all light levels, and both were similar to sham in this regard for illumination levels greater than 0 lux. The

additional cohort of impact TBI mice will eventually provide more insight into the effects of impact TBI on light sensitivity at 70 and 115 days, and on any sham mice anxiety at 115 days.

Subtask 1-1: Functional and Morphological Studies of Blast TBI

3D. Raloxifene Benefit – Pupil Light Reflex after Blast TBI. To evaluate the pupillary light response (PLR), we used a Melan-100 instrument (BioMed Vision Technologies, Ames, IA). Mice are awake, and held using minimal manual restraint during the pupil light response recording session, after having been previously habituated by extensive handling. The experiments are carried out under scotopic conditions. The Melan-100 has two diode-based light sources: 630 nm for red light (200 kcd/m2) and 480 nm for blue light (200 kcd/m2). Red light elicits rod–cone-mediated PLR, and does not activate the photopigment melanopsin of intrinsically photosensitive retinal ganglion cells (ipRGCs). Blue light elicits a combined rod–cone and ipRGC response during the light, with the ipRGC response yielding a PLR sustained after light offset. An image of baseline pupil diameter is taken in the dark using an infrared video camera (Sony Handycam; Sony Corporation). A 2-second light stimuli is used to illuminate one eye at a time at a distance of 4 cm, beginning with the red light. The pupil responses are recorded with a digital infrared camera. Prior to blue light illumination, pupils are allowed to recover for ten seconds. Video movies are captured of the mouse eyes prior and during red light stimulation, during the 10-second interval prior to blue light, during blue light, and ten seconds afterward blue light offset. Frames from the captured movies are analyzed using image analysis software to measure pupil size before, during and after the light stimulus. These studies were carried out at 25 weeks (175 days) after blast, and were completed during the second year of the project, and involve analysis of both eyes in 14 sham-vehicle mice, 14 blast-vehicle mice, 16 blast-5mg/kg raloxifene mice, and 10 blast-10mg/kg raloxifene mice.

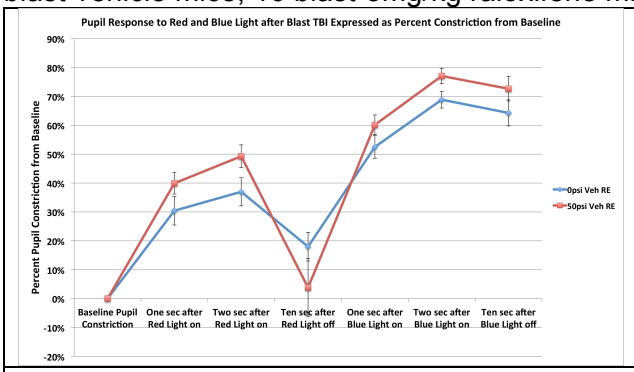


Figure 5A. Effect of blast TBI (with only vehicle treatment) on right eye pupil constriction in response to red and blue light. Pupil responses are plotted as % constriction (i.e. pupil area reduction) from pre-illumination baseline. Pupil constriction is significantly enhanced in the 50V mice to both red and blue light.

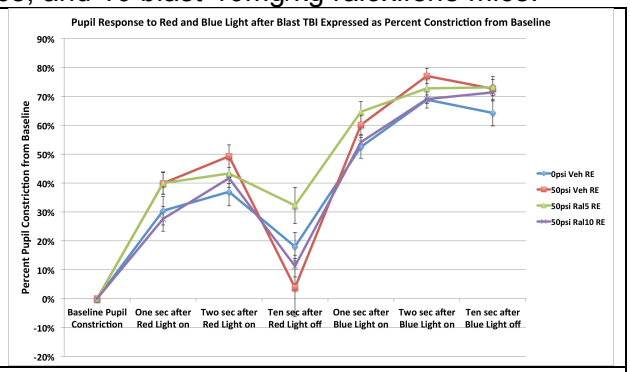


Figure 5B. Effect of blast TBI on right eye pupil constriction in response to red and blue light. The enhanced pupil constriction in the 50V mice to red light is rescued by both raloxifene doses and the enhanced pupil constriction in the 50V mice to blue light is rescued by the higher but not lower raloxifene dose.

Of particular interest, we found abnormalities in the pupil response of the right eye for the 50V mice both when exposed to red light and when exposed to blue light (Figure 5A). In the case of the red light exposure for the right eye, pupil constriction was significantly enhanced during the red light by the Tukey post ANOVA comparison (p=0.050) and tended to be less constricted 10 seconds after red light offset in the 50V mice than in sham mice, thus indicating some abnormal hyper-responsiveness in the photoreceptors driving the pupil light response to red light, or in their coupling to the ganglion cells driving the pupil light response. More notably, the right eye in the 50V mice also showed a significantly increased response to blue light (Figure 5A) from pre-blue light baseline that persevered after light off compared to the 0V mice, that was significant by the Tukey post ANOVA comparison (p=0.025). The increased pupil responsiveness may be linked to the increased light aversion see in our 50V mice, as both may

be caused by an over-reaction to light. The red light but not the blue light response was normalized in the 50-psi mice treated with 5 mg/kg raloxifene (Figure 5B), since it was not significantly different than for control 0V right eyes for the red light (p=0.226) but was greater for blue light (p=0.014). The 10 mg/kg dose of raloxifene, however, normalized the exaggerated red and blue light constriction seen in the 50V mice, since neither the response to the red light (p=0.997) or the blue light (p=0.798) differed from the sham mice. Thus, the higher raloxifene dose was less effective overall than the 5 mg/kg dose in preventing the exuberant right eye pupil light reflex caused by the 50-psi blast TBI.

Somewhat surprisingly, we did not observe any abnormality in the pupil light reflex of the left eye in the case of the 50-psi blast vehicle-treated mice with either red or blue light (Figure 6A). It may be that whatever mechanism caused the hyper-pupil responsiveness in the right eye was counteracted for the left eye by the greater optic nerve axon loss (as detailed below) we tend to see for the left eye after TBI. Notably, however, the left eyes in the 50-psi mice treated with 10 mg/kg raloxifene showed a consistently and significantly poorer pupil constriction for the red light (p=0.006) but not the blue light (p=0.248). Thus, although 10 mg/kg raloxifene was either beneficial (right eye) or harmless (left eye) for the ipRGC-driven blue light driven PLR, the photoreceptor-driven pupil response for the left eye after TBI was adversely affected. As the pupil response is mainly is mainly ipRGC driven, any adverse effect of the higher raloxifene dose for photoreceptor-driven pupil constriction is outweighed by its normalizing effect on the pupillary over-reaction to red or blue light after blast TBI. Moreover, our ERG A-wave data noted above revealed no abnormalities in the photoreceptor response of blast mice treated with a dose of 10 mg/kg raloxifene.

Figure 6A. Effect of blast TBI (with only vehicle treatment) on left eye pupil constriction in response to red and blue light. Pupil responses are plotted as % constriction (i.e. pupil area reduction) from pre-illumination baseline. Pupil constriction is the same in the 0V and 50V mice to both red and blue light.

Figure 6B. Effect of blast TBI on left eye pupil constriction in response to red and blue light. Pupil constriction in the 50V mice treated with the higher but not lower raloxifene dose is significantly diminished in response to red light, but not significantly different than sham mice in response to blue light.

Subtask 2-1: Functional and Morphological Studies of Impact TBI

3D. Raloxifene Benefit – Pupil Light Reflex after Impact TBI. We evaluated Light-Dark Box behavior for three groups of impact TBI mice at 28 weeks (196 days) post-TBI during the second project period: sham – vehicle mice (n=6), impact TBI – vehicle mice (n=9), and impact - raloxifene mice 5 mg/kg (n=5). As the impact injury is at the midline, the eyes, optic nerves, and two sides of the brain are equally affected. In the case of the pupil response, as expected, the left and right eyes did not differ. Thus, data are presented and analyzed for the two eyes pooled within group. In the case of the pupil constriction to red light, we observed that the response in 50-psi blast vehicle-treated mice was significantly reduced compared to sham impact mice during the red light, most notably at the 2 second mark (Figure 7), as assessed by the Tukey post ANOVA comparison (p=0.001). By contrast, the pupil constriction during red light in the impact TBI mice treated with raloxifene did not differ significantly from that in the sham mice (p=0.689), although the pupil tend to return closer to baseline after red light offset in

the impact TBI mice treated with raloxifene. In any case, these results indicate that the photoreceptor-mediated pupil constriction to red light was diminished by impact TBI and at least partly rescued by raloxifene. By contrast, the response to blue light was indistinguishable during and after blue light from sham in the case of the impact-vehicle mice (p=0.184), but significantly less than in sham during blue light in the case of impact-raloxifene mice (p=0.005). The blue light response (which is normally sustained even after light offset) in the impact-raloxifene mice, however, was the same as in sham 10 sec after blue light off. Thus, the pupil response to blue appeared sluggish but normal in its ultimate amplitude in the impact-raloxifene mice. Overall, our findings for the pupil light reflex show a differing outcome with our blast and impact TBI. Left side cranial blast TBI enhances the pupil response to red and blue light, at least for right eye, while dorsal midline cranial impact TBI reduces the constriction to red light. We do not know if this reflects an inherent difference between blast and impact as concussive forces, or (more likely) to differences between the site of impact (lateral left cranial for blast and dorsal midline cranial for impact) or between the magnitude of the concussive forces. In any case, raloxifene normalized the response to red light after impact TBI, but slowed the response to blue light. As noted, a second cohort of impact mice has been created and studied for contrast sensitivity and acuity, and their pupil responses will be assessed in May.

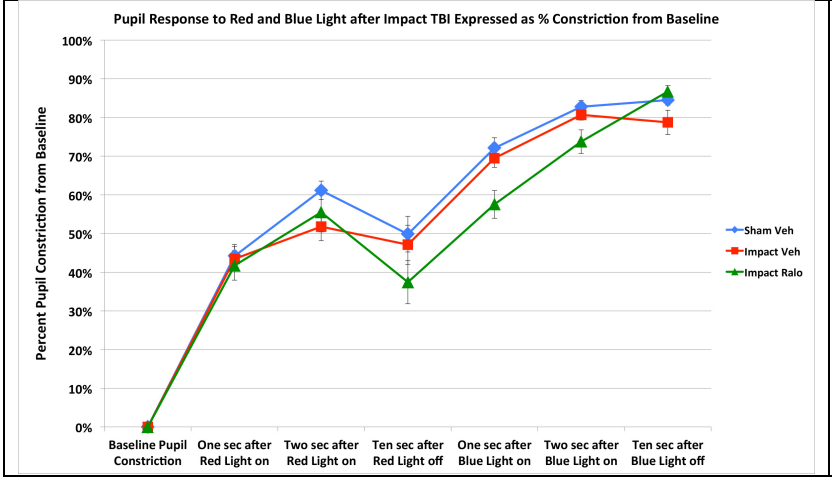


Figure 7. Effect of impact TBI (with only vehicle treatment) on the pooled left eye and right pupil constriction in response to red and blue light. Pupil responses are plotted as % constriction (i.e. pupil area reduction) from pre-illumination baseline. Pupil constriction is significantly less in the impact-vehicle mice than in the sham-vehicle mice to red light, but no different in response to blue light. Raloxifene after TBI normalizes the response during red light but diminishes it during blue light, although the sustained blue light response is normal after blue light offset.

Subtask 1-1: Functional and Morphological Studies of Blast TBI

3E. Morphological Benefit of Raloxifene in Blast TBI. We have evaluated raloxifene benefit after TBI in brain and retina. A detailed description focusing on progress during the second year of the project follows.

Raloxifene Benefit in Mice with Blast TBI – Retina and Optic Tract. We have quantified the benefit of 5 mg/kg raloxifene for retina and right optic tract 3-6 days after blast TBI in a cohort of 15 mice, and at 30-50 days after blast TBI in a cohort of 12 mice during the second year of the project. As shown in the graphs below, at both 3-6 days (Figure 8A) and 30-50 days (Figure 8B) after blast TBI, the number of IBA1+ microglia in the optic tract was significantly increased bilaterally – by 92.3% at 3-6 days and by 69.1% at 30-50 days. The increase in microglia largely reflected an increase in microglia with an activated, amoeboid morphology. We believe that microglial activation is in reaction to the optic axon injury that occurs in our blast TBI model. We have found that this optic axon injury can be revealed by SMI32 immunostaining, which shows many pathological axon bulbs bilaterally in the optic tract at 3-6 days post TBI. In the case of the IBA1 immunostaining, we found that the bilateral optic tract increase was reversed by raloxifene at either a 5 or 10 mg/kg dose. This presumably reflects an alteration in the activation state of the microglia away from the M1 state. Analysis of injured axons in the optic tract in these same cases, as evaluated by detecting fragmented axon bulbs using immunostaining for SMI32, indicated that the IBA1+ microglia reduction was associated with a

50% reduction in damaged axons. In the case of retina 3-6 days post blast, we observed a significant 31.1% bilateral increase in IBA1+ microglia in 50-psi blast animals not receiving raloxifene post-blast, which was reversed by 5 mg/kg raloxifene (Figure 8C).

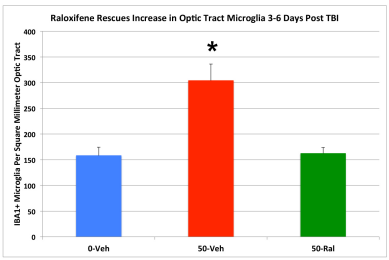


Figure 8A. Rescue of bilateral increase in optic tract microglia 3-6 days after blast TBI with 5mg/kg raloxifene.

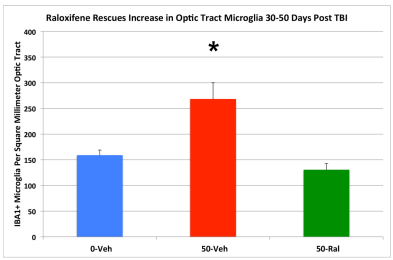


Figure 8B. Rescue of bilateral ncrease in optic tract microglia 30-50 days after blast TBI with 5mg/kg raloxifene.

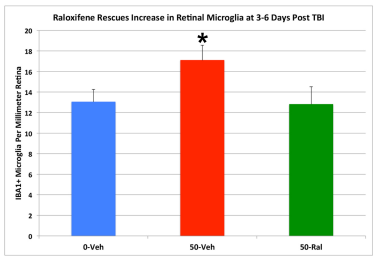


Figure 8C. Rescue of bilateral increase in retinal microglia of the eye 3-6 days after blast TBI with 5mg/kg raloxifene.

Raloxifene Benefit in Mice with Blast TBI – Optic Nerve. For 40 blast mice for which functional assessment of raloxifene benefit in blast TBI is complete, we harvested optic nerves and embedded them in plastic. Optic nerves were then sectioned at 1 micron, stained with phenylenediamine (PPD), mounted on slides, and blinded computer-assisted axon counts performed. For the right optic nerve (RON) no significant axon loss was seen in the 50 psi-vehicle mice, but there was a trend toward an ~5% reduction in the left optic nerve (LON). A reduction of similar magnitude was seen for the 50-psi 5 mg/kg raloxifene mice, while the mean axon count for the 50-psi 10 mg/kg raloxifene mice was very similar to that in the sham mice. Although this analysis currently appears underpowered for ANOVA with post-hoc tests (typically currently only 10 per group), we did find that the axon abundance across all left optic nerves was significantly correlated with contrast sensitivity for those eyes (r=0.368). Thus, the reduction in left optic nerve axons in the 50-psi vehicle mice may have contributed to the poorer contrast sensitivity. We will analyze the optic nerves of a second cohort of blast TBI mice now that their functional testing is complete, which will increase the number of optic nerves analyzed per side to 15-16 per group. This is likely to provide more statistical clarity as to whether axon loss occurs in the left optic nerve in 50-psi vehicle mice, and any rescue occurs in the 50-psi 5 mg/kg raloxifene mice. These additional optic nerves are currently being embedded and sectioned, and will be analyzed this summer.

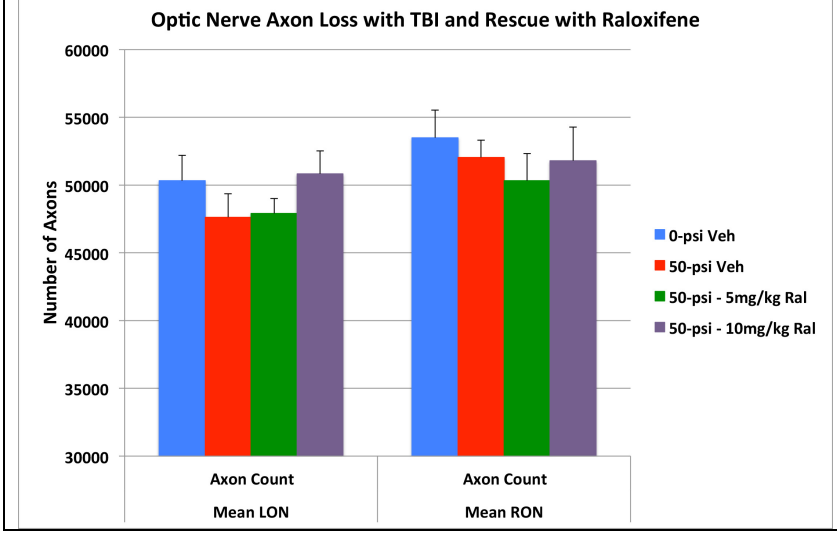


Figure 9. Blinded computer-assisted optic nerve axon counts for first cohort of blast mice. The analysis seems underpowered, but a suggestion of axon loss is seen for the left optic nerve but not the right in mice receiving blast TBI and vehicle. Raloxifene at 10 mg/kg rescues the apparent loss for the left optic nerve after blast. The optic nerve axon abundance is significantly correlated with the contrast sensitivity score for the left eye, suggesting that the ~5% loss of axons in the left optic nerve contributed to this functional deficit. An additional cohort will shortly be analyzed to increase statistical power.

Raloxifene Benefit in Mice with Blast TBI – Oculomotor Nuclei. As part of our goal to understand the basis of eye movement abnormalities in TBI victims and assess their alleviation

by raloxifene, we have examined the oculomotor neuron pools in our TBI mice using immunolabeling for choline acetyltransferase (ChAT) to detect oculomotor neurons. The findings below were completed during year 2 of the project. We have focused on the oculomotor nucleus (cranial nerve nucleus III), because it innervates 4 extraocular muscles, and performed blinded counts of cholinergic neurons in the cell group and measurement of the area of the cell group at a matched standard level across cases. Tissue was immunolabeled for the cholinergic marker ChAT to allow unambiguous identification of oculomotor neurons. Measurements have been performed at >31 days post blast to assess loss and rescue in one set of cases, and at 3-6 days post blast to assess short term effects and the treatment window needed for rescue in another set of cases. At >31 days post-blast, neuron abundance was significantly bilaterally reduced by 25% and oculomotor nucleus area was significantly bilaterally reduced by 30% in the 50-psi vehicle-treated mice (n=9) compared to sham (n=7) (Figures 10A, 10B, 11A). Remarkably, both deficits were completely rescued in 50-psi mice (n=7) that had received 5 mg/kg raloxifene for 2 weeks (Figure 11A). At a mean of 5 days postblast, we also found that oculomotor nucleus area was bilaterally reduced by about 25% in 50-psi vehicle mice (n=10) compared to sham mice (n=7), but a 20% reduction in cholinergic neuron abundance only trended toward reduction in blast-vehicle mice. The absence of significant neuron loss at this early time point after blast suggests more time may be needed to see the loss. In additional mice, we also evaluated if raloxifene (5 mg/kg) treatment over days 0-5 (n=3) or for only days 3-5 (n=3) rescued oculomotor pathology at 5-6 days postblast. We found that raloxifene for days 0-5 rescued the area reduction, but treating for only days 3-5 did not (Figures 10C, 10D, 11B). Thus, our data indicate that oculomotor nucleus neurons are damaged by blast TBI, and they tentatively suggest that prompt and sustained daily treatment that lasts for at least five days is needed to prevent their long-term loss. These results are illustrated in the graphs below, in which asterisks indicate a significant difference from sham-vehicle.

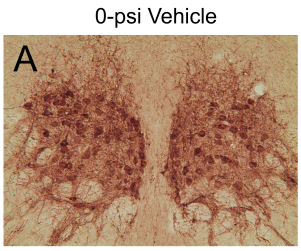


Figure 10A. Cholinergic neurons of the left and right oculomotor nuclei in a mouse 6 days after sham blast.

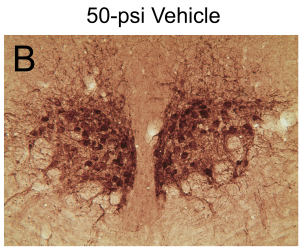


Figure 10B. Cholinergic neurons of the left and right oculomotor nuclei in a mouse 6 days after 50-psi blast.

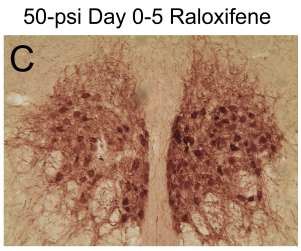


Figure 10C. Cholinergic neurons of the oculomotor nuclei in a mouse 5 days after 50-psi blast with daily raloxifene.

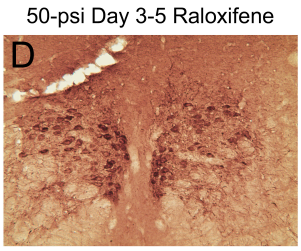


Figure 10D. Cholinergic oculomotor neurons 5 days after 50-psi blast treated with raloxifene only for the last 3 days.

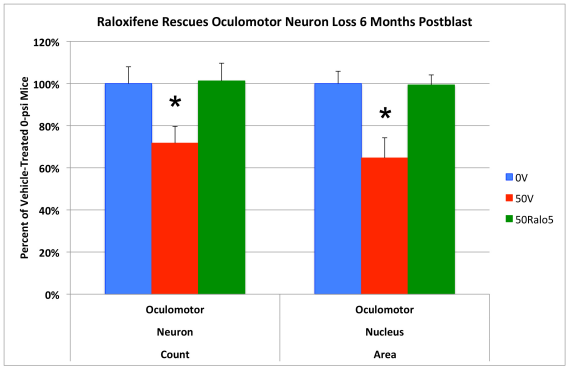


Figure 11A. Loss of oculomotor neurons >1 month post blast and their rescue with raloxifene.

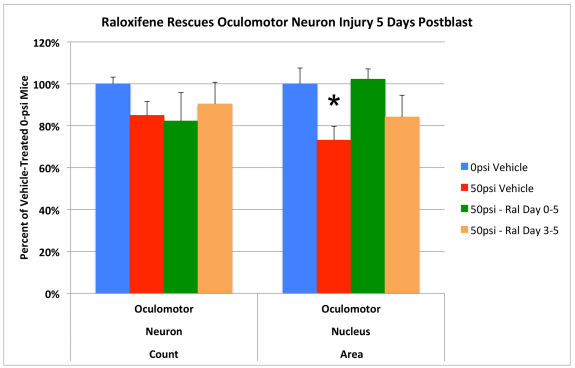


Figure 11B. Pathology of oculomotor neurons a mean of 5 days post blast and their rescue with raloxifene.

Raloxifene Benefit in Mice with Blast TBI – Retina. As functional assessment of raloxifene benefit in blast TBI is completed, we harvest retinas from these cases for morphological analysis. We plan to analyze these retinas as flat-mounts so we can perform pan-retinal analysis of any regional photoreceptor injury (using immunolabeling for rod or cone markers), any amacrine cell injury (using the cholinergic starburst amacrine cells as the exemplar), any loss of retinal ganglion cells (RGCs) as suggested likely by optic nerve axon loss for at least the left eye (using Brn3a immunolabeling as the readout), and any change in the abundance or melanopsin expression of intrinsically photoreceptive retinal ganglion cells (ipRGCs) to determine if increased melanopsin expression accounts for the enhanced pupillary light reflex seen in 50-psi blast vehicle-treated mice. Multiple immunofluorescence labeling and confocal laser scanning microscopy (CLSM) will be used to scan and capture images for analysis. Retinas will typically be simultaneously labeled for a cone marker (S-opsin), for a marker of cholinergic amacrine cells (choline acetyltransferase, ChAT), for Brn3a to detect RGCs, and for melanopsin to detect ipRGCs. Using different fluorophores and/or different depths of focus, we will be able to distinguish these different cell types, as shown in the images below. In some cases, we may additionally or alternatively evaluate rods using a rod marker (rod arrestin or rhodopsin). Prior to processing the retinas from the experimental animals, we have used non-experimental mice to successfully refine our methods for harvesting retinas, immunolabel them for these markers, place them on slides as flat mounts, and capturw images of the entire flat mount retina or localized retinal regions, at different retinal depths in the tissue so we can visualize the different cell types. Figure 12 below shows several images that illustrate the type of multiple immunolabeling and images we have obtained and will use to evaluate the effect of TBI on retinal cell types and the rescuing effect of raloxifene. Note the regular array of cholinergic amacrine cells and RGCs in Figures 12A and 12B, which are from the same field of view visualized with different fluorophores. This retina was also immunolabeled for melanopsin, with the same ipRGCs evident in both 12A and 12B. If TBI disrupts cholinergic amacrine cells or RGCs, it will be evident as a gap in the array and be quantifiable as a reduction in numbers in blinded computer-assisted counts. In the case of the ipRGCs, Figure 12C shows that we will be able to label them with sufficient clarity to use the intensity of melanopsin immunolabeling to determine if there is an increase in ipRGCs in the right eyes of 50-psi blast mice that might explain the enhanced pupil response.

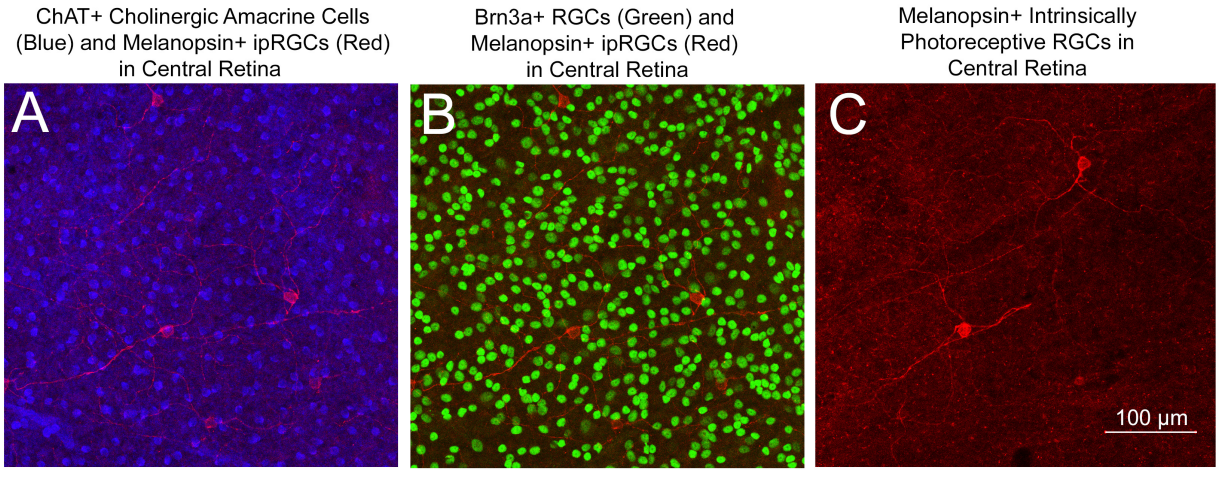


Figure 12. Flat mounted mouse retinas showing immunofluorescence labeling in a single field of view in images A and B, and a different field of view in C, as detected using a Zeiss 710 confocal laser scanning microscope. Cholinergic amacrine cells (Blue) and ipRGCs (Red) are shown in A, and RGCs (Green) and the same ipRGCs are shown in B. Image C shows melanopsin+ ipRGC alone (Red) to illustrate the clarity and degree of their labeling, including their dendritic processes.

Figure 13 below shows a flat mounted mouse retina immunolabeled and visualized using a Zeiss 710 confocal laser scanning microscope for rod arrestin (red) and S-opsin (green).

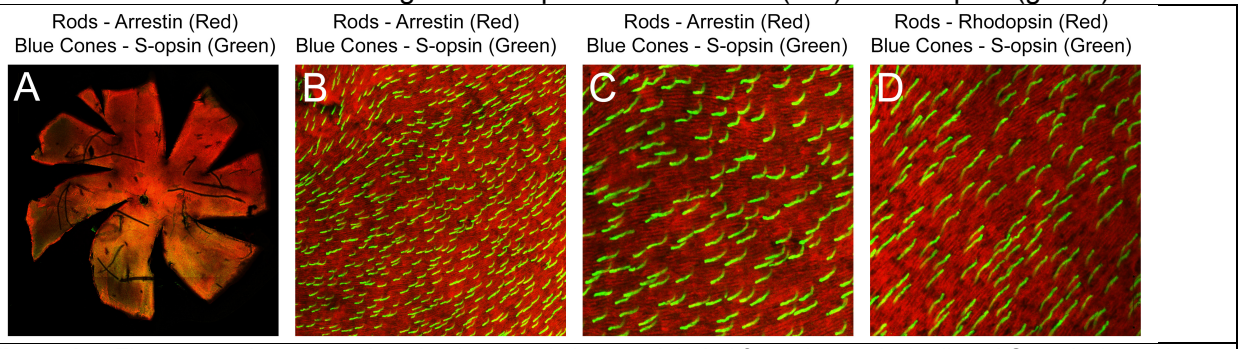


Figure 13. Flat mounted mouse retina simultaneously immunolabeled for rod arrestin (red) and S-opsin (green), and visualized using a Zeiss 710 confocal laser scanning microscope. Image A is at low magnification to view entire flat mount. B and C show successively higher magnification views of central retina. D shows a field of view from a different retina in which rod outer segments are immunolabeled for red rhodopsin, and cones green for S-opsin.

S-opsin containing cones are sparse in dorsal retina and abundant ventrally. Thus, the red labeling of upper retina in Figure 13A reflects predominant rod labeling, while the more orange labeling ventrally reflects a mix of red rod and green cone labeling. The rod versus cone labeling at successively higher magnification in images B and C shows the regular interdigitization of rod and cone outer segments, with rods far more abundant. Image D shows a similar field of view as in C, except in this case rod outer segments are immunolabeled for rhodopsin. Images such as those in Figure 13 will reveal if blast causes alterations to photoreceptor cell types, and if raloxifene rescues this.

Subtask 2-1: Functional and Morphological Studies of Impact TBI

3E. Morphological Benefit of Raloxifene in Impact TBI. We will evaluate raloxifene benefit morphologically after impact TBI in brain, retina, and optic nerve using the above described approaches for blast TBI during the first part of the third year. Tissues have been harvested from the first impact cohort, and in the case of retinas embedded in plastic.

Subtask 1-2: Studies of Microglia Modulation in Blast TBI

3G. Raloxifene Benefit in Mice with Blast TBI – Biochemical Studies. We developed and refined our protocols for assessing microglial modulation by blast TBI and raloxifene, and conducted studies on a cohort of mice. As part of this, we developed our methods for harvesting mRNA from retina, optic nerve and visual thalamus (target of retinal input), and performing reverse transcription (RT) to convert this to cDNA. We also developed our methods

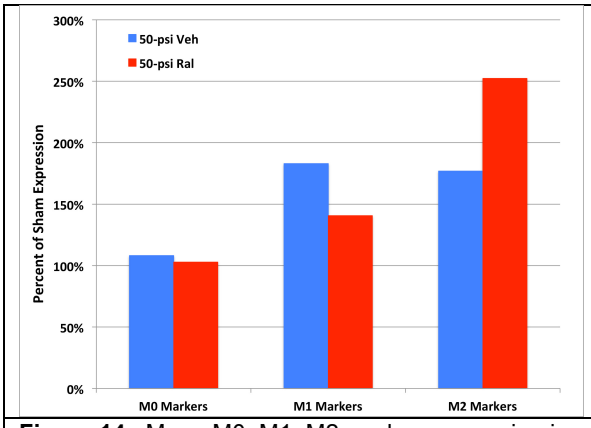


Figure 14. Mean M0, M1, M2 marker expression in thalamus for blast-vehicle and blast-raloxifene mice, compared relative to sham expression.

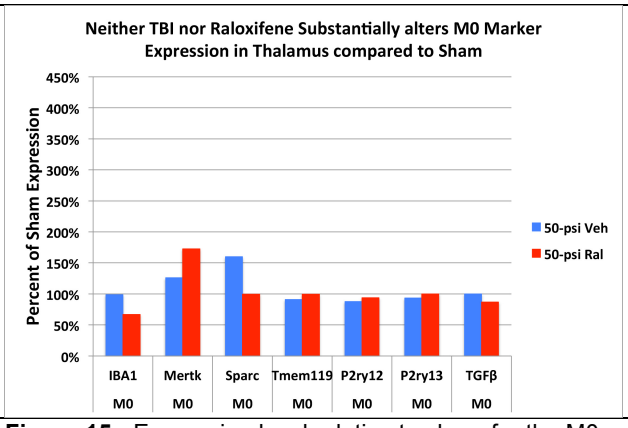


Figure 15. Expression level relative to sham for the M0 markers in thalamus for blast-vehicle and blast-raloxifene mice.

for linearly amplifying cDNA so that we have adequate amounts for using qPCR to detect transcripts that distinguish among the M0, M1 and M2 states of microglia, especially in the case of tissues such as the optic nerve and retina, from which the mRNA harvest is low because of limited volume of tissue. We identified 21 transcripts to assess the M0, M1, and M2 states, and obtained primers for them. These transcripts are: 1) M0 – IBA1, Mertk, Tmem119, P2ry12, P2ry13, SPARC, TGFβ; 2) M1 - CD16/32, IFNγ, IL1α, IL1β, IL6, TNFα, iNOS, IBA1; and 3) M2 - CD206, Arginase-1, YM1, IL10, TREM2. Using these markers and the above described approach, we evaluated microglial modulation in the thalamus by blast TBI and raloxifene in a set of 3 sham mice, 3 blast-vehicle mice, and 3 blast-raloxifene (5 mg/kg) mice, all sacrificed 3 days after blast. Our qPCR analysis (Figure 14) showed that blast plus vehicle yielded an overall comparable elevation in M1 and M2 marker expression, increasing them to about 75% more than in sham mice. By contrast, raloxifene reduced M1 marker expression and increased M2 marker expression relative to the blast-vehicle mice. Overall, the M2 – M1 marker ratio was 0.97 for the blast-vehicle thalamus and 1.80 for the blast-raloxifene mice, thus showing that raloxifene did bias microglia expression toward the M2 profile. Figure 15 shows the expression level relative to sham for the M0 markers. Note that expression was similar to that in sham for most M0 genes for both the blast-vehicle and blast-raloxifene. Figures 16 and 17 below show how blast and raloxifene affected the relative expression of individual M1 and M2 markers. Three days after blast, all M1 markers showed increased expression relative to sham blast mice, except for CD16 and IL1α, and raloxifene reduced expression of all M1 markers except for TNFα. Raloxifene increased expression of 4 of the 5 M2 markers examined, and for three of these (arginase-1, Trem2, and IL10) the levels were increased far above that in blast-vehicle mice. Our qPCR results to date are thus consistent with the view that blast increased M1 microglial activation (and M2 as well), and raloxifene biased microglia after blast away from the M1 and toward the M2 state, as consistent with our expectations based on its CB2 receptor inverse agonism. During the third year of the project, additional mice will be analyzed to obtain a sufficient number of mice per group for statistical assessment.

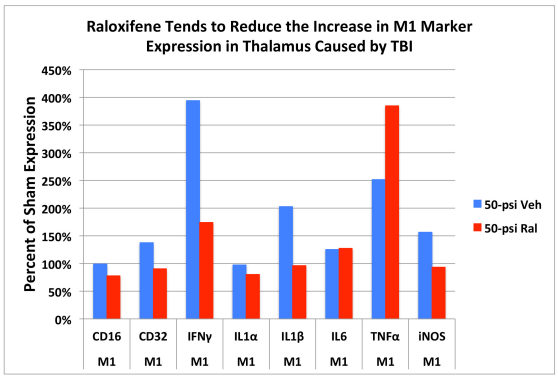


Figure 16. M1 marker expression in thalamus for blast-vehicle and blast-raloxifene mice, compared relative to sham expression.

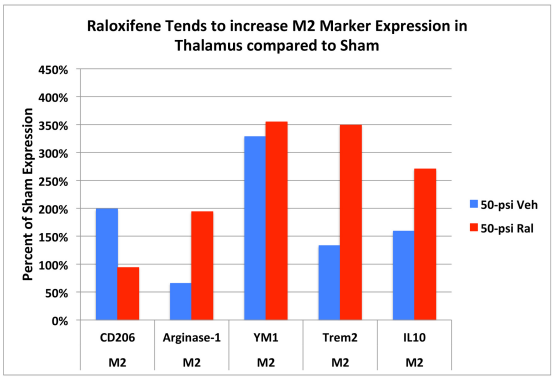


Figure 17. M2 marker expression in thalamus for blast-vehicle and blast-raloxifene mice, compared relative to sham expression.

Subtask 2-2: Studies of Microglia Modulation in Impact TBI. These studies will be undertaken during year 3 of the project, once the blast studies are complete.

Subtask 1-3: Raloxifene Treatment Window for Blast TBI - Effective Treatment Window for Functional Benefit. We studied several groups of blast mice during year two to evaluate if delayed treatment with raloxifene yields benefit in treating visual deficits after TBI. In one group (n=5), mice did not begin to receive raloxifene (5 mg/kg) until the day after 50-psi blast (thus missing treatment on day 0, the day of blast), and then received daily raloxifene until 14 days

post blast. Mice in a second group (n=5) did not begin to receive raloxifene (5 mg/kg) until the second day after 50-psi blast (thus missing treatment on day 0, the day of blast, and day 1, the day after blast), and then received daily raloxifene until 14 days post blast. Both groups of mice were pre-blast tested for visual contrast sensitivity and visual acuity to establish their baselines, and then received testing for visual contrast sensitivity and visual acuity one month after blast. These two groups represent part of our effort to determine the effective treatment window for raloxifene, with two window groups having been previously studied during year 1 of the project – a group of 5 mice that did not begin to receive raloxifene (5 mg/kg) until the third day after 50-psi blast (thus missing treatment on days 0-2) and then received daily raloxifene until 14 days post blast, and a group of 5 mice that only received 4 daily treatments after 50-psi blast TBI (days 0-3). The results for all four groups with different treatment regimens were compared to those obtained for the 15 vehicle-treated sham mice, 16 vehicle-treated 50-psi blast TBI mice, and 17 raloxifene (5mg/kg)-treated 50-psi blast TBI mice (some of which were completed in year 2). Evaluating contrast sensitivity for the entire data set (Figure 18), we found that raloxifene could be delayed until the second day after blast and still eliminate the left eye contrast sensitivity deficit caused by 50-psi blast. Delaying until the third day, or only treating over days 0-3 did not prevent the left eye contrast sensitivity deficit. The right eye contrast sensitivity deficit was prevented by all of these treatment regimens, indicating that the pathogenic process leading to the right eye contrast sensitivity deficit must differ from that for the left eye (and presumably not involve early optic axon injury as for the left eye), since it was rescued by brief or delayed raloxifene treatment. This is consistent with our optic nerve count data, since the left eye but not the right eye contrast sensitivity deficit correlates with axon loss after blast TBI. The right eye contrast sensitivity deficit apparently is related to a more delayed or slow inflammatory effect that even delayed raloxifene treatment can arrest, and we do not know the extent to which the right eye deficit is centrally or peripherally mediated. With regard to visual acuity (Figure 19), we found that raloxifene could be delayed until the third day postblast, or only delivered over days 0-3, and still prevent the right eye acuity deficit. As acuity loss is not correlated with optic nerve axon loss for that eye, it seems likely the acuity deficit stems from brain or retinal injury not requiring prompt or sustained raloxifene treatment. Our data indicate, however, that the left eye contrast sensitivity deficit requires more prompt and sustained raloxifene (i.e. within ~48 hours and lasting until day 14 post blast), but optimal benefit is still obtained if treatment is not begun until the second day after blast (~48 hours after blast). The results for contrast sensitivity and visual acuity are illustrated in the below graphs (Figures 18 and 19). Asterisks in all cases for the below figures indicate a significant deficit for that eye and that vision test.

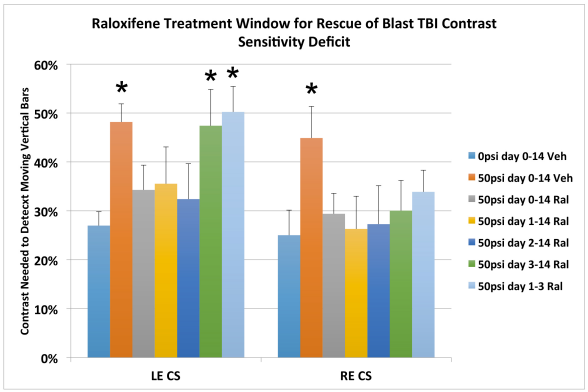


Figure 18. Contrast sensitivity performance after blast and different raloxifene treatment regimens.

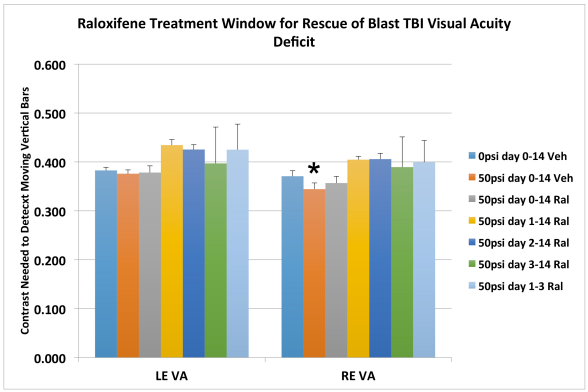


Figure 19. Visual acuity performance after blast and different raloxifene treatment regimens.

Subtask 1-3: Raloxifene Treatment Window for Blast TBI – Effective Treatment Window for Morphological Benefit in Optic Tract. We also used a morphological approach to

evaluate if delaying treatment with 5 mg/kg raloxifene reduces or eliminates its benefit. For these studies, we relied on our finding that blast TBI to the left side of the cranium produces left optic nerve damage that in the brain manifests as axon pathology in the right optic tract and microglial activation over the first 3-6 days after blast. The axon pathology occurs as axonal swellings called bulbs that can be detected with SMI32 antibody labeling, while the microglial activation involves an increase in microglia detectible by immunolabeling for IBA1. For these studies, we assessed if delaying raloxifene treatment until the third day after 50-psi blast (and then continuing treatment until sacrifice on day 5 postblast) reduced the benefit seen with treatment beginning 2 hours after TBI and continuing for 5 days, both in terms of IBA1+ microglia in the optic tracts and SMI32+ axon bulbs in the optic tracts. The results are shown in the graphs below (Figures 20A, B). Blast plus vehicle yielded significant increases in IBA1+ microglia over that seen in sham – a doubling for the left optic tract and a quadrupling for the right optic tract (Figure 20A). The microglial activation is greater for the right optic tract because the axon injury is greater for the left optic nerve, which crosses in the brain to become the right optic tract. For the left optic tract (LOT), microglia remained significantly increased in the blast mice receiving 5 mg/kg raloxifene daily from the outset. Surprisingly, no significant increase was seen for the LOT in the mice with delayed treatment. For the right optic tract (ROT), IBA1+ microglia were significantly increased above sham for both the daily and the delayed raloxifene, but daily raloxifene significantly reduced microglia below the abundance seen in the blast-vehicle mice (Figure 20A). Thus, for the ROT, the current data suggest that delaying raloxifene reduced its benefit, though some benefit may have been retained. A similar result was seen in the case of axon bulb pathology (Figure 20B). Axon bulb pathology is absent in normal optic tract, and was slightly but significantly increased in the LOT in blast-vehicle mice. Lesser but still significant increases were observed in the LOT in blast mice with either daily or delayed raloxifene. A much larger increase in axon bulbs was seen for ROT in the blast-vehicle mice, and lesser but still significant increases were observed with both daily and delayed raloxifene. Notably, daily raloxifene significantly reduced axon bulbs below the abundance seen in the blast-vehicle mice. These overall results suggest that delayed raloxifene yielded a lesser benefit than daily raloxifene, but some benefit nonetheless.

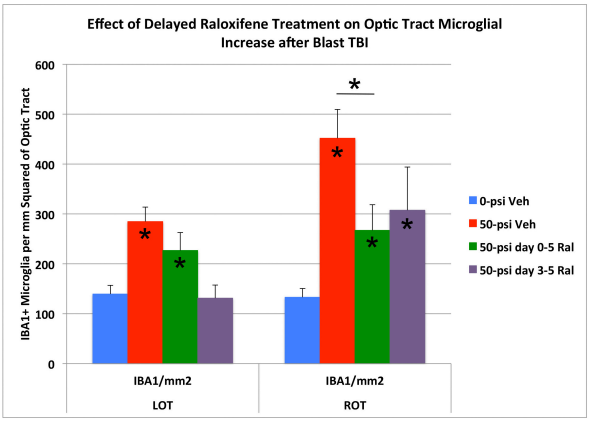


Figure 20A. Rescue of bilateral increase in optic tract microglia 5 days after blast TBI with continuous versus delayed 5mg/kg raloxifene.

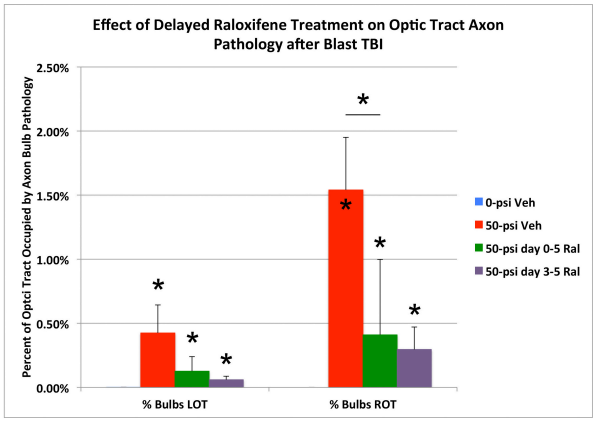


Figure 20B. Rescue of bilateral increase in optic tract axon bulbs 5 days after blast TBI with continuous versus delayed 5mg/kg raloxifene.

Subtask 1-3: Raloxifene Treatment Window for Blast TBI - Effective Treatment Window for Morphological Benefit in Oculomotor Nuclei. As discussed above in the section on the rescue with raloxifene of oculomotor nucleus pathology after blast TBI, we found that raloxifene for days 0-5 rescued oculomotor nucleus pathology, but treating for only days 3-5 did not (Figures 10C, 10D, 11B). Thus, our data indicate that oculomotor nucleus neurons are damaged by blast TBI, and prompt and sustained daily treatment that lasts for at least 5 days is

needed to prevent their long-term loss. Together with our functional studies, our morphological results show that delaying raloxifene until the third day post blast significantly reduces but does not entirely eliminate the benefit.

Subtask 2-3: Treatment Window for Impact TBI. We anticipate that the lessons learned about the treatment window needed for raloxifene benefit for blast TBI will also be relevant to impact TBI. Nonetheless, we will specifically examine this during year 3 of the project.

4) Other Achievements.

Light – Dark Box Studies – Anxiety following Blast TBI. Two-chamber tests, with one of the chambers being enclosed by dark walls and the other open, are typically used as tests of anxiety. In this test, an increased tendency to remain in the enclosed chamber reflects an anxiety increase. We found that 50V mice showed a significant increase in time spent in the enclosed chamber at both 0 lux and 500 lux illumination by chi-square compared to 0V mice ($p=0.0277$) in the testing performed at 100 days after blast. The 500 lux illumination of the enclosed chamber was apparently not bright enough to override this anxiety, although 1000 lux was. Raloxifene at both doses rescued this anxiety. Although not part of our original goals, we present this unexpected finding because of the interest of the DOD in not only treatments for vision problems after TBI but also for neuropsychiatric problems. These results suggest that raloxifene treatment after TBI may mitigate anxiety caused by the TBI. We did not see this same anxiety in the second round of testing in the light-dark box in which we evaluated behavior in 500, 1000, and 1500 lux, presumably because the earlier testing had habituated mice to the apparatus and thereby reduced the anxiety evident in it.

Task 1: What opportunities for training and professional development has the project provided? The project was not intended to provide training and professional development opportunities, and so there is Nothing to Report.

Task 2: What opportunities for training and professional development has the project provided? The project was not intended to provide training and professional development opportunities, and so there is Nothing to Report.

Task 1: How were the results disseminated to communities of interest? We are engaging in final collecting of data on blast TBI and we plan to publish our findings during the 2018 calendar year.

Task 2: How were the results disseminated to communities of interest? We are still collecting data on impact TBI and so it is premature to seek to publish our findings. Thus, there is Nothing to Report.

Task 1: What do you plan to do during the next reporting period to accomplish the goals?

1. We will complete analysis of the morphological benefit of raloxifene for retina and optic nerve following blast TBI.
2. We will complete analysis of the effect of raloxifene on microglia following blast TBI.

Task 2: What do you plan to do during the next reporting period to accomplish the goals?

1. We will complete analysis of the function benefit of raloxifene for ERG, light sensitivity, and the pupil response following impact TBI.
2. We will complete analysis of the morphological benefit of raloxifene for retina, optic nerve and optic tract following impact TBI.
3. We will complete analysis of the effect of raloxifene on microglia following impact TBI.

4. We will complete studies to determine the necessary and sufficient treatment window for raloxifene following impact TBI.

Impact

What was the impact on the development of the principal discipline(s) of the project?
Due to our evidence for a robust benefit of raloxifene in mitigating visual deficits and anxiety after blast TBI, and mitigating visual deficits after impact TBI, we have initiated planning a randomized phase 2 clinical trial in collaboration with Dr. Martin Croce, trauma surgeon at UTHSC and Medical Director of the Elvis Presley Memorial Trauma Center at Regional One Health and Dr. Marcia Honig, a co-investigator on the current award. A large community of researchers and clinicians at UTHSC is dedicated to the study and treatment of head trauma, and the Elvis Presley Memorial Trauma Center is one of the busiest trauma centers in the country, with about 5,000 annual trauma admissions. Approximately 15% of these blunt injured patients suffer a traumatic brain injury. The PIs of the Phase 2 Clinical Trial (Drs. Reiner, Honig, and Croce) will work with several of Dr. Croce's clinician colleagues in developing the protocols and recruitment strategy for a phase 2 trial of raloxifene efficacy for visual and cognitive deficits after head trauma. We recently responded to a request by the Department of Defense (DoD) Defense Health Program (DHP) Joint Warfighter Medical Research Program (JWMRP) managed by the Office of Congressionally Directed Medical Research Programs (CDMRP) for information about the status of our project. The JWMRP provides an opportunity to advance previously-funded DoD or Service medical R&D projects that address the medical requirements of the Services and the Military Health System. We described our progress, indicated our plans for the phase 2 trial, and noted our funding needs for this trial in our response to the JWMRP.

What was the impact on other disciplines?
Nothing to Report.

What was the impact on technology transfer?
Nothing to Report.

What was the impact on society beyond science and technology?
Nothing to Report.

Changes/Problems

Changes in approach and reasons for change.
There have been no changes in approach, objectives or scope during the reporting period. Thus, Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them.
During the first three months of the project (period 1), delays were encountered for several reasons. First, we could not purchase animals or raloxifene until the grant had started. As a result, the start of animal testing was delayed a few weeks until the receipt of mice (April 4) and raloxifene (March 31). Two members of our research staff (Del Mar and Li) were moved into two of the staff positions on this project, and upon the start of the project the process of hiring to fill the third staff position was initiated and completed, with Desmond Henderson joining our team on June 7. Additionally, because the first set of mice did not show substantial deficits in contrast sensitivity with 50-psi blast, we needed to devote time to recalibrate our blast system.

Although the initial delays slowed the project, the first task is nearly complete. The impact studies of the second year were delayed in their start until June 2017, which was the soonest that our consultant Dr. Rad Tzekov could visit to instruct us on use of the impact TBI system. Progress was also unavoidably slowed because one of the staff conducting these studies, Dr. Nobel Del Mar, suffered a severe left should injury after he slipped and fell on ice in January 2018. This impaired his work performance, and he ultimately went on disability leave and had rotator cuff surgery in early March, and is expect back in late May 2018. We expect to make up for any delays during the third year of the project, and during a no-cost extension subsequent to that.

Changes that had a significant impact on expenditures.
There have been no changes that have had a significant impact on expenditures. Nothing to Report.

Significant changes in use or care of human subjects.
No human subjects are used. Nothing to Report.

Significant changes in use or care of vertebrate animals.
Nothing to Report.

Significant changes in use of biohazards and/or select agents.
No biohazards or select agents are used. Nothing to Report.

Products

Publications, conference papers, and presentations
We are engaging in final collecting of data on blast TBI and we plan to publish our findings during the 2018 calendar year.

Website, Technologies/Techniques, Inventions, Patent Applications, Licenses, and Other Products
Nothing to Report.

Participants and other Collaborating Organizations

What individuals have worked on the project?

Name:	Anton Reiner
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1.2
Contribution to Project:	Dr. Reiner has overseen the research and interpreted results.

Name:	Marcia Honig
Project Role:	co-Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1.2

Contribution to Project:	Dr. Honig has aided in overseeing the research and interpreting results.
Name:	Bob Moore
Project Role:	co-Investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	0.6
Contribution to Project:	Dr. Moore formulated the raloxifene for in vivo testing in mice.
Name:	Nobel Del Mar
Project Role:	Technical Director
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6.0
Contribution to Project:	Dr. Del Mar performed the TBI and animal testing.
Name:	Chunyan Li
Project Role:	Research Associate
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	6.0
Contribution to Project:	Dr. Li performed TBI and animal testing.
Name:	Desmond Henderson
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	12.0
Contribution to Project:	Desmond performed TBI and animal testing.
Name:	Hongbing Wang
Project Role:	Research Associate
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2.5
Contribution to Project:	Hongbing analyzed microglia biochemistry.
Name:	Aaron Perry
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2.0
Contribution to Project:	Aaron analyzed microglia biochemistry.
Name:	Tyler Ragsdale
Project Role:	Medical Student
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2.5
Contribution to Project:	Tyler counted optic nerve axons.
Name:	Andrew Fortugno
Project Role:	Medical Student
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2.5
Contribution to Project:	Andrew analyzed microglia biochemistry.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report.

What other organizations were involved as partners?
Nothing to Report.

Special Reporting Requirements

COLLABORATIVE AWARDS: The current award is not a collaborative award, so there is Nothing to Report.

QUAD CHARTS: An updated Quad Chart is attached to this submission.

Appendices

No appendices at this time.

CB2 Receptor Therapy Using the FDA-approved Drug Raloxifene to Mitigate Visual Deficits after Mild TBI and/or Ocular Trauma

Funding Opportunity Number: W81XWH-14-CRMRP-NSRRA

PI: Anton Reiner **Org:** University of Tennessee Health Science Center **Requested Amount:** $1,346,882

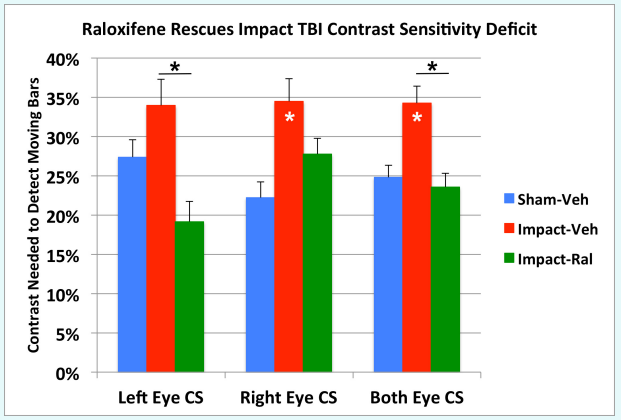
Study Aim

•Visual disabilities after brain and/or closed-globe ocular trauma from blast injury are highly common in military personnel, but effective treatments are lacking. We have found that early post-injury treatment with CB2 cannabinoid receptor inverse agonists mitigates visual impairments caused by mild TBI in a preclinical model.

•We hypothesize that the recently discovered CB2 receptor inverse agonism of raloxifene, which is FDA approved for treatment of osteoporosis, will make it effective for mitigating visual disabilities after brain and/or closed-globe ocular trauma. We will test its effectiveness in doing so in preclinical models.

Approach

We will test the efficacy of raloxifene for reducing retinal and optic nerve damage and visual deficits stemming from mild TBI in two different standardized mouse models and in closed-globe ocular injury in mice.



Example of Raloxifene benefit: Rescue of contrast sensitivity deficit after dorsal midline cranial impact TBI. Data shown are the contrast needed to see the moving bars – with increased contrast needed reflecting a deficit.

Accomplishment: We found that raloxifene administered daily after focal cranial blast TBI in mice reduces visual deficits and visual system pathology, and ongoing studies show a similar benefit in focal cranial impact TBI in mice.

Timeline and Cost

Activities	CY16	CY17	CY18
Confirm visual benefit of raloxifene in a blast model of mild TBI			
Confirm visual benefit of raloxifene in an impact model of mild TBI			
Confirm visual benefit of raloxifene in a model of ocular blast injury			
Estimated Total Budget ($K)	Dir: $285K ID: $146K	Dir:$305K ID: $153K	Dir: $301K ID: $157K

Updated: (4/12/2018)

Goals

- **Study 1 (CY16)**: We will confirm the effectiveness of the newly discovered CB2 receptor inverse agonism of raloxifene in mitigating visual deficits in our standardized blast model of mild TBI in mouse. As part of these studies, we will refine dose and timing of treatment, and perform mechanistic studies to demonstrate the role of microglial modulation in the benefit.
- **Study 2 (CY17):** We will confirm the effectiveness of raloxifene in mitigating visual deficits in a standardized impact model of mild TBI in mice.
- **Study 3 (CY18):** We will confirm the effectiveness of raloxifene in mitigating visual deficits in a standardized model of ocular blast injury in mice.

Comments/Challenges/Issues/Concerns

- None.

Budget Expenditure to Date

Projected Expenditure: $590,000

Actual Expenditure: ~$560,000